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Effect of levels and sources of dietary manganese on growth and mineral composition of post-smolt Atlantic salmon fed low fish meal, plant-based ingredient diets

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ABSTRACT

The impact of dietary manganese (Mn) levels and sources on the growth and mineral composition of post-smolt Atlantic salmon (*Salmo salar*) fed practical diets was studied. Seven experimental diets were prepared with graded supplementation level of Mn; basal diet had a Mn concentration of 15 mg kg^{-1}, four diets with 5, 15, 35 and 65 mg kg^{-1} supplementation of Mn as $MnSO_4$ and two diets with at 5 and 15 mg kg^{-1} supplementation of Mn as Mn-glycine (Mn-gly). Atlantic salmon (initial weight, 307 ± 25 g) were distributed to 21 tanks (35 fish/tank). These fish were randomly fed with one of the experimental diets, in triplicate groups to apparent satiation for 8 weeks. At week 4 and 8, samples of whole body, plasma, bile, liver and vertebrae were collected, and their mineral concentration determined. At week 8, feaces were collected by stripping for measuring apparent availability coefficient (AAC). Neither the Mn inclusion level, nor the source had a significant impact on growth and other performance indicators. Plasma and bile Mn concentrations were significantly affected by dietary treatments at both sampling points, at week 4 and 8; whereas, whole body, liver and vertebrae responded significantly only at the end of 8 weeks. Dietary Mn level needed to meet the requirement of post-smolt Atlantic salmon was estimated by non-linear regression models using saturation of whole body or tissue Mn status as response criteria. The estimates based on Mn concentration in whole body, vertebrae, plasma and apparent availability were 29.5 ± 5.3, 26.2 ± 2.7, 26.3 ± 4.9 and 34 ± 3.4 mg Mn kg^{-1} diet, respectively. Analysis of relative bioavailability index showed that low inclusion of Mn-gly (5 mg kg^{-1} supplementation) was 2.6 to 4.5-fold more efficient than $MnSO_4$ to attain whole body or tissue Mn saturation levels. On the contrary, high inclusion of Mn-gly (15 mg kg^{-1} supplementation) reduced the AAC of Mn, zinc (Zn) and copper (Cu), along with lower Mn and Zn status in tissues. The mean estimate of dietary Mn required to maintain tissue Mn saturation ranged between 26 and 34 mg Mn kg^{-1} diet; the corresponding Mn supplementation (as $MnSO_4$) ranged from 14 to 22 mg Mn kg^{-1} diet. The supplementation level can be reduced to 4.9 to 5.7 mg Mn kg^{-1} diet by using Mn-gly as Mn source without compromising growth or Mn status of Atlantic salmon. High inclusion levels of Mn-gly (15 mg kg^{-1} diet) was found to be not beneficial.

1. Introduction

Manganese (Mn) is a micro-mineral essential for growth, vertebral development and anti-oxidant metabolism in fish (Watanabe et al., 1997; Lall and Lewis-McCrea, 2007). Diet is the main source of Mn supply and the entero-hepato-biliary system plays a major role in regulating the absorption and excretion of Mn in vertebrates (Britton and Cotzias, 1966). In fish, although the regulation of Mn metabolism is not well understood, the effect of Mn deficiency leading to dwarfism, low body and vertebrae Mn status have been well documented (Watanabe et al., 1988; Lorentzen et al., 1996; Satoh et al., 1991; Satoh et al., 1987; Gatlin and Wilson, 1984; Knox et al., 1981). Therefore, Mn supplementation is required in practical feeds to meet the Mn requirement of Atlantic salmon (*Salmo salar*). A minimal supplementation

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of 12–15 mg Mn kg^{-1} dry feed was recommended for rainbow trout juveniles and Atlantic salmon parr to maintain Mn saturation levels in whole body or vertebrae, when fed diets based predominantly on fish meal (Watanabe et al., 1988; Lorentzen et al., 1996). Given the varying levels and bioavailability of micro-minerals in practical feed ingredients (Sugiura et al., 1998), the dietary levels required are reported to vary (Antony Jesu Prabhu et al., 2018a; Welker et al., 2018). Moreover, no report is available on the dietary Mn levels required for Atlantic salmon during seawater phase.

Diet composition of Atlantic salmon feeds have changed to a great extent in the recent years with increased inclusion of plant-based ingredients, especially in feeds for seawater phase. The average fish meal inclusion in Atlantic salmon feeds in Norway during 2013 was 18% and it was projected to be reduced even further in the forthcoming years (Ytrestøyl et al., 2015). In present day, a blend of plant-based ingredients are being used in Atlantic salmon feeds to replace fish meal (Ytrestøyl et al., 2015). Among the essential micro-minerals (e.g. copper (Cu), iron (Fe), selenium (Se), zinc (Zn) and Mn), the concentration of Cu and Mn are generally higher, whereas Zn and Se are lower in plant-based ingredients than found in fish meal (Antony Jesu Prabhu et al., 2018a). However, the dietary bioavailability is highly variable, more often low due to the presence of a diverse array of anti-nutritional factors which limit the absorption of both endogenous and minerals supplemented through premix (Francis et al., 2001). Recent re-evaluations of dietary micro-mineral supplementation to plant-based ingredient diets for rainbow trout also point to low or highly variable bioavailability of endogenous Mn (Welker et al., 2018; Antony Jesu Prabhu et al., 2018a).

Mn supplementation in fish feeds is usually made using inorganic salts and the most common salt used is manganese (II) sulphate ($MnSO_4$). Alternatively, Mn chelated to one or more amino acids have gained interest in animal and fish feeds, potentially to improve the bioavailability (Silva et al., 2019; Nie et al., 2016). The principle underlying improved bioavailability of the chelated mineral sources is that, (i) binding of the mineral (Mn, in this case) to an amino acid will reduce the susceptibility of the mineral to negative interactions by anti-nutritional factors like phytic acid (Silva et al., 2019); and (ii) it will improve intestinal uptake by enterocytes as it can open up alternate uptake pathways in addition to the divalent metal transporters (Zhang et al., 2017). In this context, two research aims were addressed in this study: (i) evaluation of the dietary Mn supplementation level for Atlantic salmon in seawater, when fed low fish meal diets; (ii) comparative evaluation of an inorganic ($MnSO_4$) and chelated (Mn-glycine) form as Mn source in low fish meal Atlantic salmon feeds.

2. Material and methods

2.1. Design of the experiment

The study was designed to establish optimal Mn supplementation level and evaluate the efficiency of Mn-glycine (Mn-gly) as Mn source in comparison with $MnSO_4$. To establish the Mn supplementation level, a standard single factorial regression with five graded supplementation levels of Mn was used. Two additional treatments supplemented with chelated Mn-gly at place of $MnSO_4$ at two inclusion levels were also included. The treatments were performed in triplicate groups.

2.2. Experimental diets

The basal diet was intended to closely represent the commercially used feed formulations for seawater Atlantic salmon in Norway. Nevertheless, ingredients were chosen to be as low in Mn as possible and similar to the diet formulation used in our previous study to examine the apparent availability of mineral sources (inorganic, IM or organic, OM) in seawater Atlantic salmon (Silva et al., 2019). The basal diet was then formulated to contain no > 15 mg Mn kg^{-1} diet (Table 1). Five different diets IM0, IM5, IM15, IM35 and IM65 were then prepared from the basal diet with graded inclusion levels of Mn (0, 5, 15, 35 and 65 mg Mn kg^{-1} diet, as manganous sulphate monohydrate ($MnSO_4.H_2O$, Mn 32%, Vilomix, Hønefoss, Norway) to obtain targeted total nominal levels of 15, 20, 35, 50 or 80 mg Mn kg^{-1} diet. Two other diets with inclusion of 5 (OM5) or 15 (OM15) mg Mn kg^{-1} diet as Mn-gly (as Mn chelate of glycine hydrate ($Mn(x)_{1-3}.nH_2O$, x = anion of glycine ($C_2H_4NO_2^-$), Mn 22%, Phytobiotics, Eltville, Germany) were also prepared. All the seven diets were isocaloric and isonitrogenous (Skretting ARC, Norway). The analysed Mn concentration in the basal diet was 15 mg kg^{-1} diet; the four IM diets ($MnSO_4$ supplemented) were analysed to contain 19, 34, 46 and 79 mg Mn kg^{-1} diet; and the two Mn-gly supplemented diets had 20 and 33 mg Mn kg^{-1} diet (Table 2).

Table 1
Formulation and composition of the basal experimental diet.

Ingredients (%)	
Whole wheat	8.3
Corn gluten	15.0
Hi-pro soya	14.4
Wheat gluten	20.0
Soy protein concentrate	10.0
Fish meal[a]	5.0
Fish oil[a]	9.9
Rapeseed oil[b]	12.3
Micro-ingredients and premixes[c]	5.4
Proximate composition (analysed, $n = 7$)	
Dry weight (%)	92 ± 0.1
Energy (kJ/g)	23.5 ± 0.1
Lipid (%)	25 ± 0.4
Protein, analysed as N * 6.25 (%)	45 ± 0.5
Ash (%)	4.5 ± 0.3
Phosphorus (%)	0.9 ± 0.02
Calcium (%)	0.6 ± 0.02

[a] North-Atlantic.
[b] European, non-GM.
[c] Contains monoamonium phosphate, histidine HCl, yttrium oxide, L-lysine and DL-methionine and astaxanthin; standard vitamin and mineral mix, excluding manganese.

2.3. Fish, husbandry and feeding

Seven hundred and thirty five post-smolt Atlantic salmon (mean initial weight, 307 ± 25 g), were distributed among 21 tanks at the rate of thirty-five fish in each tank with a mean initial biomass of 10.75 ± 0.09 kg. The tanks were supplied with flow-through seawater with salinity of 33 ppt and a temperature range of 10–12 °C. The 7 experimental diets were randomly assigned to the 21 tanks in triplicates. The fish were fed the experimental diets twice a day to apparent satiation for 8 weeks. The uneaten feed pellets were collected to estimate the actual feed intake.

2.4. Sampling and analytical procedures

The weight and length of the fish were recorded at the start and after 4 and 8 weeks of experimental feeding period after euthanasia by an overdose (6 mL L^{-1}) of tricaine methanesulphonate (PharmaQ, Bergen, Norway). Samples for proximate composition and mineral analysis were taken at start and at the end of week 4 and 8. Homogenised pooled samples of whole body (10 fish/tank), liver (6 fish/tank), and vertebrae (6 fish/tank) and individual samples of plasma, bile, liver (aliquot from same 6 fish used for pooled samples above) were collected. Plasma, bile and vertebrae were stored at −20 °C; whereas, the liver samples were flash frozen in liquid nitrogen and further stored at −80 °C until analysis. In addition, at the end of week 8, faeces were collected by stripping from all fish and pooled per

Table 2
Study design, manganese (Mn) supplementation and analysed concentration of Mn in the experimental diets.

Mn source	Diet code	Mn inclusion (mg kg^{-1} diet)	Desired dietary Mn levels (mg kg^{-1} diet)	Analysed Mn level in diets (mg kg^{-1} diet)	Supplementation with ref. to NRC[a] & EC[b]
Basal diet	Basal	0	15	15	Low[a]
Inorganic source ($MnSO_4$)	IM5	5	20	19	Sub-optimal[a]
	IM15	15	30	34	Adequate[a]
	IM35	35	50	46	Above requirement[a]
	IM65	65	80	79	Near maximum limit[b]
Organic source (Mn-gly)	OM5	5	20	20	Sub-optimal[a]
	OM15	15	30	33	Adequate[a]

Data available on the Mn requirement of Atlantic salmon in freshwater (NRC, 2011; Lorentzen et al., 1996) and maximum permitted concentration for total Mn in complete fish feeds with 12% moisture (European Commission, 2017; European Commission, 2003) were used as reference to determine Mn supplementation levels to be tested in this study. The Mn requirement, 10–15 mg kg^{-1} diet on available basis; Maximum limit, 100 mg kg^{-1} diet.

[a] NRC, National Research Council.

[b] European Commission (2017), European Commission (2003).

tank for apparent availability measurements. Faeces samples were freeze dried for 72 h at −80 °C, homogenised with a pestle and mortar into a fine powder and stored at room temperature until further analysis. The diets were homogenised and analysed for estimating the dry matter, ash, lipid, protein following standard procedures. Briefly, dry matter was measured after drying at 103 °C for 24 h; ash content determined by combustion in a muffle furnace at 550 °C for 16–18 h; lipid was determined following ethyl-acetate and acid-extraction in fish tissue and feeds, respectively (Lie, 1991); protein (6.25 x nitrogen) was measured with a nitrogen analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Germany) according to AOAC official methods of analysis (Sweeney and Rexroad, 1987). Haematocrit were measured as described in Antony Jesu Prabhu et al. (2017). The concentration of Mn, Cu, Zn, Se and Fe in diets, faeces and tissues were analysed by inductively coupled plasma mass spectrometry (ICP-MS) according to Julshamn et al. (1999); and, yttrium in diets and faeces samples were analysed as described in Silva et al. (2019).

2.5. Regression models and calculations

The determination of the required level of Mn inclusion, three non-linear regression models namely (i) BL1, broken-line with plateau (Robbins et al., 2006); (ii) BL2, broken-line with two lines (Robbins et al., 2006); and (iii) QP, quadratic plateau (Simongiovanni et al., 2012) were applied to the data from five dietary groups (basal diet and four graded inclusion of Mn as MnSO4), and the best-fit model was selected based on R^2 value. Apparent availability coefficient (AAC) and retention were calculated according to the formulae described in Antony Jesu Prabhu et al. (2014). The relative bioavailability index of Mn-gly compared to $MnSO_4$ was calculated considering the percentage saturation in specific response criteria achieved with 5 mg Mn supplementation kg^{-1} diet as Mn-gly, adapted from Antony Jesu Prabhu et al. (2018a).

2.6. Data analysis

The data are presented as mean and pooled standard deviation, using tanks as the experimental units for all parameters analysed ($n = 3$). One-way ANOVA at significance level of 0.05, followed by Tukey's multiple comparison analysis was used to determine the effect of dietary treatments. In the case of non-normal distribution of data, Kruskal-Wallis non-parametric test was used. Area under the curve analysis was used to identify the peaks in bile and plasma Mn concentrations. All the data analysis was performed using GraphPad Prism version 8.0 for Windows, GraphPad Software, California USA.

3. Results

3.1. Growth performance indicators

Atlantic salmon fed the different experimental diets more than doubled their body weight in the period of 8 weeks with no differential effect of the Mn levels or sources (Table 3). The mean weight gain of the fish was 320 ± 15 g (mean ± SD); with the highest recorded in OM5 fed fish (336 ± 10 g) and the lowest in fish fed diet with OM15 diet (305 ± 6 g). None of the performance indicators examined were significantly different between the treatments (Supplementary table 1). The mean ± SD across all the groups for specific growth rate, condition factor, percentage feed intake per day, feed efficiency and hepatosomatic index was 1.4 ± 0.03, 1.4 ± 0.02, 1.1 ± 0.03, 1.28 ± 0.03, and 1.16 ± 0.03, respectively. No mortality was observed.

3.2. Macro-nutrient and mineral composition of whole body

The proximate composition of whole body was not influenced by dietary treatments at any stage of the experiment, neither at week 4 nor at week 8. At the end of week 8, dry matter, protein, fat, ash and energy content were not significantly different between the treatments averaging 35 ± 0.1%, 17 ± 0.1%, 15 ± 0.1%, 2 ± 0.1% and 9.9 ± 0.1 kJ/g, respectively (Supplementary table 2). Similarly, the final whole body concentration of phosphorus, calcium, magnesium, sodium and potassium were respectively 4.7 ± 0.4, 4 ± 0.7, 0.37 ± 0.2, 0.87 ± 0.3 and 3.9 ± 0.1 g kg^{-1} and were also not differentially affected by the Mn levels or sources (Supplementary table 3).

Table 3
Growth performance of Atlantic salmon fed different dietary levels and sources of manganese (Mn) for 8 weeks in seawater.

Mn source	Mn Inclusion level (mg kg^{-1})	Body weight (g)		Weight gain (g)		Specific growth rate (% day^{-1})	
		Wk 4	Wk 8	Wk 4	Wk 8	Wk 4	Wk 8
Basal diet	0	454.2	633.4	148.4	327.6	1.62	1.42
$MnSO_4$	5	451.7	620.8	145.7	314.8	1.59	1.38
	15	450.8	621.5	143.7	314.5	1.57	1.37
	35	456.5	624.6	147.1	315.8	1.62	1.38
	65	454.6	633.7	147.2	326.3	1.62	1.42
Mn-gly	5	454.5	643.8	147.1	336.5	1.62	1.45
	15	443.8	611.7	137.2	305.0	1.53	1.35
Pooled SD		6.9	16.2	5.9	15.3	0.07	0.03
p-value		ns	ns	ns	ns	ns	ns

Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis; ns, treatment effects not statistically significant, $p > 0.05$. Wk, sampling week. Mean initial weight of the fish, 307 ± 26 g.

Table 4
Whole body and tissue manganese (Mn) concentration of Atlantic salmon fed different dietary levels and sources of Mn for 8 weeks in seawater.

Mn source	Mn inclusion level (mg kg^{-1})	Whole body		Vertebrae		Liver	
		Wk 4	Wk 8	Wk 4	Wk 8	Wk 4	Wk 8
Basal diet	0	1.3	1.0^{a}	10.1	9.5^{a}	1.4	1.4^{a}
$MnSO_4$	5	1.1	1.3^{ab}	11.0	11.7^{ab}	1.5	1.5^{a}
	15	1.4	1.7^{b}	11.7	12.7^{b}	1.5	1.5^{a}
	35	1.6	1.5^{ab}	12.3	13.3^{b}	1.6	1.6^{b}
	65	1.5	1.8^{b}	12.0	13.7^{b}	1.6	1.7^{b}
Mn-Gly	5	1.6	1.5^{ab}	13.7	13.7^{b}	1.7	1.5^{a}
	15	1.0	1.7^{b}	12.7	12.7^{b}	1.6	1.5^{a}
Pooled SD		0.32	0.18	1.6	0.7	0.09	0.05
p-value		ns	0.004	ns	0.0004	ns	0.001

All data presented as mg per kg wet weight. Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > 0.05$. Wk, sampling week.

Table 5
Haematocrit (Hct), plasma and bile manganese (Mn) concentration of Atlantic salmon fed different dietary levels and sources of Mn for 8 weeks in seawater.

Mn source	Mn inclusion level (mg kg^{-1})	Hct	Plasma		Bile		Plasma:Bile	
		Wk 8	Wk 4	Wk 8	Wk 4	Wk 8	Wk 4	Wk 8
Basal diet	0	40.1	1.1	1.4^{a}	0.47^{a}	0.37^{a}	2.5	3.8
$MnSO_4$	5	45.4	1.3	2.3^{b}	0.47^{a}	0.47^{a}	3.0	5.0
	15	40.2	2.1	3.2^{c}	0.52^{a}	0.59^{ab}	4.0	5.0
	35	41.4	1.8	2.8^{bc}	0.74^{b}	0.79^{b}	2.5	3.7
	65	40.5	1.7	3.0^{c}	0.58^{a}	0.55^{ab}	2.9	5.5
Mn-gly	5	40.6	2.1	3.0^{c}	0.85^{b}	0.55^{ab}	2.4	5.3
	15	42.5	1.6	2.3^{b}	0.53^{a}	0.55^{ab}	3.1	4.3
Pooled SD		2.7	0.3	0.5	0.08	0.09	0.4	0.9
p-value		ns	0.02	0.02	0.001	0.01	ns	ns

Hct presented as %; plasma and bile Mn concentrations presented as μmol L^{-1}. Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > 0.05$. Wk, sampling week. Hct, haematocrit.

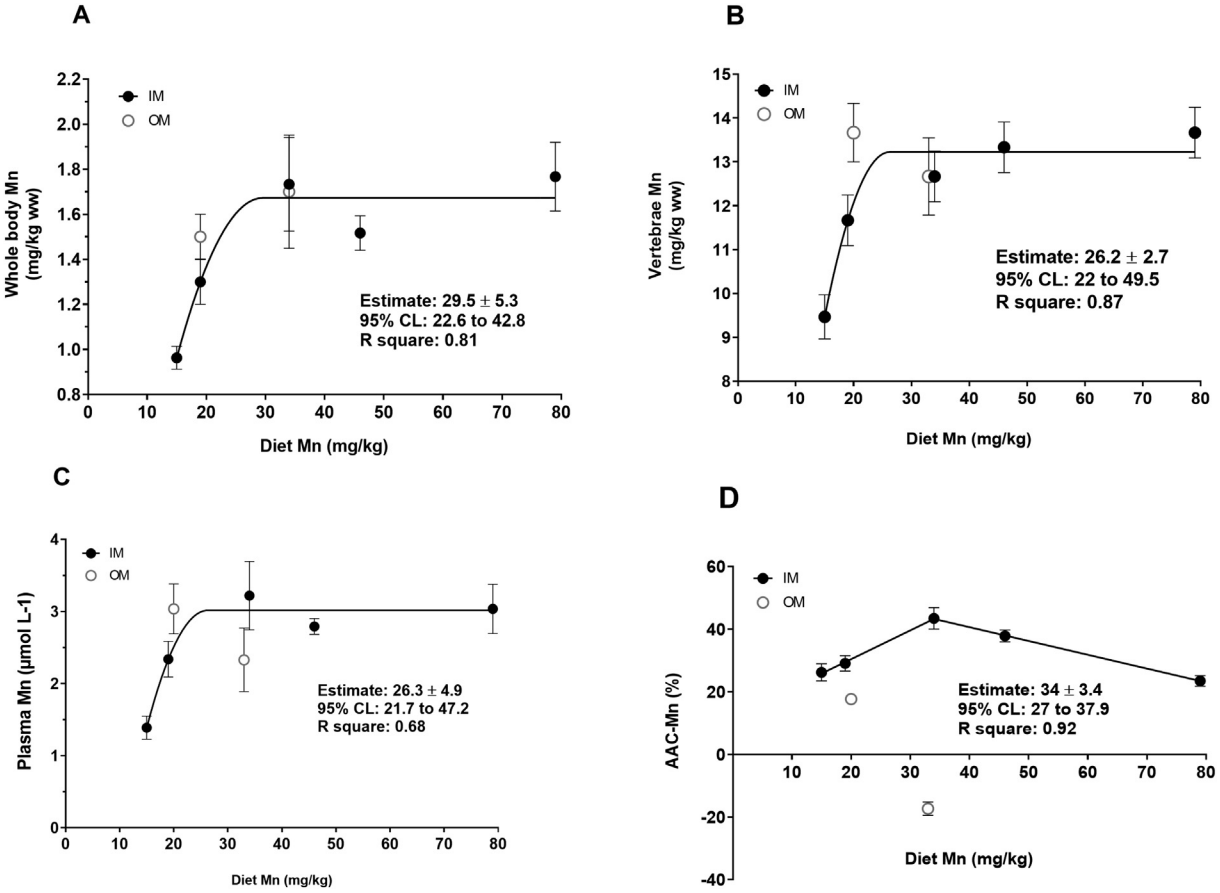

Fig. 1. Dietary Manganese (Mn) level required to reach whole body or tissue Mn saturation in seawater Atlantic salmon fed low fish meal diets.
Legend: Regression analysis of different response criteria (Y-axis) in Atlantic salmon (after 8 weeks) fed graded Mn levels in the diet (X-axis). The dark circles represent data points from MnSO4 groups (IM); while, white circles represent Mn-gly groups (OM). Manganese concentration in whole body (A), vertebrae (B), plasma (C) and apparent availability coefficient (D) are presented. Three non-linear regression models namely (i) BL1, broken-line with plateau (Robbins et al., 2006); (ii) BL2, broken-line with two lines (Robbins et al., 2006); and (iii) QP, quadratic plateau (Simongiovanni et al., 2012) were applied to the data from five dietary groups (basal diet and four graded inclusion of Mn as $MnSO_4$), and the best-fit model was selected based on R^2 value. The requirement estimates obtained from the model, along with the 95% confidence intervals and R^2 are presented in the corresponding graphs. The mean estimate of total Mn in the diet ranged between 26 and 34 Mn kg^{-1} diet; the corresponding Mn supplementation (as $MnSO_4$) ranged from 14 to 22 mg Mn kg^{-1} diet, based on the criterion used. Data are presented as mean ± standard deviation ($n = 3$).

Among the micro-minerals, whole body Mn concentration showed significantly different response to dietary treatments at week 8, but not at week 4 (Table 4). At week 8, fish fed the basal diet had the lowest whole-body Mn level ($p < 0.001$); increasing with level of Mn inclusion, and then reached a plateau (Fig. 1A). Difference in Mn source ($MnSO_4$ vs Mn-Gly) did not have a differential impact on the whole-body Mn concentration. Whole body concentration (mg kg^{-1} fresh weight) of other essential micro-minerals namely Cu, Fe, Zn and Se were 1.8 ± 0.1, 9.6 ± 0.3, 26.8 ± 1.4 and 0.16 ± 0.005, respectively and were similar between the treatments.

3.3. Tissue manganese concentrations

Manganese concentration in vertebrae, liver, plasma and bile of Atlantic salmon at the start, after 4 and 8 weeks of feeding the experimental diets are presented in Tables 4 and 5. Manganese concentration in all the analysed tissues of fish fed the basal diet after 8 weeks of feeding was significantly lower compared to all other diets ($p < 0.05$). Vertebrae Mn concentration in Atlantic salmon fed graded Mn inclusion levels followed a curvilinear regression (Fig. 1B). Also, the fish fed OM5 diet had a significantly higher Mn status in the vertebrae compared to those fed the un-supplemented diet. The Mn concentration in the liver of Atlantic salmon at week 4 was not significantly different between the groups and was not affected by Mn level or source. However, at week 8, a significant linear relation was observed in liver Mn with increasing Mn supplementation. The Mn concentration in the plasma and bile of Atlantic salmon were significantly altered by dietary Mn concentration, both at week 4 ($p < 0.05$) and week 8 ($p < 0.05$). Among other micro-minerals, Zn concentration in plasma, liver and vertebrae after 8 weeks of feeding were significantly ($p < 0.05$) affected by Mn level and source (Table 6). Plasma Zn increased with increasing Mn supplementation in $MnSO_4$ groups and the highest plasma Zn concentration was observed in fish fed IM65 diets. Among Mn-gly fed fish, OM5 treatment had the highest plasma Zn, which was 40% higher than corresponding group fed IM5 diet; whereas, it decreased markedly in OM15 groups. The AAC of Zn was significantly higher in basal diet and it was reduced by 5 mg inclusion of $MnSO_4$, but not by Mn-gly. Apart from AAC of Cu, the concentration of Cu in whole body, liver, vertebrae, plasma and faeces were not significantly affected by Mn level or source (Table 7). Except for fish fed the IM5 diet, the AAC of Cu was significantly reduced by Mn inclusion and was the lowest in OM15.

3.4. Faecal manganese loss, apparent availability coefficient and retention

The Mn concentration in the faeces, apparent availability and retention were all influenced by dietary treatments (Table 8). Faecal Mn concentration increased linearly with increasing Mn supplementation irrespective of the Mn source ($p < 0.0001$). Zn concentration in the faeces was significantly higher in IM5 fed fish compared to other IM supplemented groups ($p < 0.05$, Kruskal-Wallis non-parametric test). The AAC of Mn followed a non-linear response (Fig. 1D) and was significantly higher in $MnSO_4$ supplemented diets ($p < 0.05$). Retention of dietary Mn was significantly higher in Atlantic salmon fed with diets IM5 and OM5, irrespective of the source ($p < 0.0001$).

3.5. Estimation of dietary manganese inclusion level

Non-linear regression analysis using a curvilinear plateau or broken line model to estimate optimal dietary Mn inclusion level is presented in Fig. 1. Whole body (Fig. 1A), vertebral (Fig. 1B), plasma Mn concentrations (Fig. 1C) and AAC (Fig. 1D) were used as response criteria. Modelling of data from $MnSO_4$ supplemented groups showed that, Mn saturation of whole body, vertebrae, plasma and apparent availability occurred at dietary Mn inclusion level of 16.3 mg Mn kg^{-1} diet (total 29.5 ± 5.3 mg kg^{-1} diet), 22.1 mg kg^{-1} diet (total, 26.2 ± 2.7 mg kg^{-1} diet), 14.1 mg Mn kg^{-1} (total, 26.3 ± 4.9 mg kg^{-1} diet) and 17.8 mg Mn kg^{-1} diet (total, 34 ± 3.4 mg kg^{-1} diet), respectively. Parameter estimates (requirement and plateau) obtained through the non-linear regression from graded inclusion of $MnSO_4$ were used to calculate the relative bioavailability index of Mn-gly (Table 9). The relative bioavailability index of Mn-gly was 2.6 to 4.5-fold higher compared to $MnSO_4$ to reach saturation levels of Mn in plasma, whole body and vertebrae. Overall, required Mn supplementation level (as $MnSO_4$) for Atlantic salmon in seawater fed low fish meal, plant-based diets range from 14.1 to 22.1 mg kg^{-1}; the corresponding supplementation levels for Mn-gly as the Mn source range from 4.9 to 5.7 mg kg^{-1} diet.

4. Discussion

In an era of rapidly changing diet compositions in commercial fish feeds, translating the requirement estimates into practical dietary recommendations is essential. The requirement of a species for a nutrient is defined as the physiological demand for maintenance, growth and reproduction. Practical dietary recommendation on the other hand refers to the dietary level of the nutrient needed to meet the requirement and thus recommendation must take into consideration the bioavailability and dietary interactions that can influence the absorption or utilisation of the target nutrient. In this context, the nutrient requirement recommendations by NRC (2011), based on data representing near 100% availability needs to be complemented with practical recommendations. Practical ingredients, like fish meal and plant-based ingredients in fish feeds can reduce trace mineral availability (Satoh et al., 1991; Yamamoto et al., 1983). For example, whilst 7.5 mg Mn kg^{-1} diet satisfied the Mn requirement of Atlantic salmon (Maage et al., 2000), supplementation of 15 mg Mn kg^{-1} diet (as $MnSO_4$) was

Table 6
Whole body, tissue concentrations and apparent availability coefficient (AAC) of zinc (Zn) in Atlantic salmon fed different dietary levels and sources of manganese (Mn) for 8 weeks in seawater.

Mn source	Mn inclusion level (mg kg^{-1})	Whole body Zn	Liver Zn	Plasma Zn	Vertebrae Zn	Faeces Zn	AAC Zn (%)
Basal diet	0	27.7	21.3^{ab}	179.6^{a}	36^{ab}	47.7^{b}	41.6^{b}
$MnSO_4$	5	26.3	21.3^{ab}	175.2^{a}	37.7^{ab}	51.0^{c}	36.9^{a}
	15	27.3	21.4^{ab}	205.4^{a}	37.7^{ab}	43.0^{a}	38.5^{a}
	35	26.2	22.3^{ab}	190.8^{a}	38.6^{ab}	41.6^{a}	40.1^{b}
	65	27.7	23.0^{b}	223.9^{b}	39.3^{b}	44.3^{ab}	41.7^{b}
Mn-gly	5	27.0	22.7^{ab}	252.6^{b}	36.6^{ab}	46.0^{b}	41.8^{b}
	15	25.7	20.6^{a}	178.3^{a}	34.7^{a}	46.3^{b}	36.3^{a}
Pooled SD		1.5	0.8	14.4	1.7	2.4	1.4
p-value		ns	0.02	< 0.001	0.04	0.01	< 0.001

Whole body, liver, vertebrae and faeces Zn concentrations presented mg kg^{-1} wet weight; plasma Zn as μmol L^{-1}. Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > 0.05$.

Table 7
Whole body, tissue concentrations and apparent availability coefficient (AAC) of copper (Cu) in Atlantic salmon fed different dietary levels and sources of manganese (Mn) for 8 weeks in seawater.

Mn source	Mn inclusion level (mg kg^{-1})	Whole body Cu	Liver Cu	Plasma Cu	Vertebrae Cu	Faeces Cu	AAC Cu (%)
Basal diet	0	1.8	110	11.4	0.28	4.1	36.1^{b}
$MnSO_4$	5	1.75	117	12.3	0.28	4.4	35.8^{b}
	15	1.77	110	11.9	0.28	4.0	32.5^{a}
	35	1.83	109	12.5	0.3	4.0	32.2^{a}
	65	1.85	104	11.6	0.29	4.3	33.0^{a}
Mn-gly	5	1.87	120	12.3	0.28	4.3	31.3^{a}
	15	1.83	117	11.2	0.28	4.2	24.3^{a}
Pooled SD		0.07	8.8	1.2	0.01	0.2	3.8
p-value		ns	ns	ns	ns	ns	0.03

Whole body, liver, vertebrae and faeces Cu concentrations presented mg kg^{-1} wet weight; plasma Cu as μmol L^{-1}. Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > 0.05$.

Table 8
Faecal loss, apparent availability coefficient (AAC) and retention of Manganese (Mn) from Atlantic salmon fed different dietary levels and sources of Mn for 8 weeks in seawater.

Mn source	Mn inclusion level (mg kg^{-1})	Faeces Mn (mg kg^{-1} wet weight)	AAC (%)	Retention (% total intake)
Basal diet	0	5.4^{a}	26.2^{c}	6.4^{a}
$MnSO_4$	5	8.3^{b}	29.1^{d}	10.8^{c}
	15	10.3^{b}	43.5^{f}	9.6^{b}
	35	18.7^{c}	37.9^{e}	5.7^{a}
	65	33.0^{d}	23.5^{c}	5.2^{a}
Mn-gly	5	9.3^{b}	17.7^{b}	12.5^{c}
	15	21.7^{c}	-17.3^{a}	9.0^{b}
Pooled SD		0.6	2.2	1.9
p-value		< 0.0001	< 0.0001	0.0009

Treatments effects were considered significant when $p < 0.05$ upon ANOVA followed by Tukey's multiple comparison post-hoc analysis. Different superscripts within a column indicate statistically significant difference between the groups; ns, treatment effects not significant, $p > 0.05$.

recommended when fed fish meal based practical diets (Lorentzen et al., 1996). Similarly, the need to supplement higher levels of trace minerals such as Zn, Cu and Se in salmonid feeds made of plant-based ingredients has been recognised (Read et al., 2014; Welker et al., 2018; Welker et al., 2016; Antony Jesu Prabhu et al., 2018a). In general, plant-based feed ingredients have higher levels of Mn than fish meal. However, the bioavailability can differ greatly within different plant-based ingredients. Regarding Mn levels in practical diets for salmonids, 17 mg Mn supplementation (as $MnSO_4$) to a basal diet containing 45 mg Mn kg^{-1} diet (Welker et al., 2018); and, no supplementation to a basal diet containing 88 mg Mn kg^{-1} (Antony Jesu Prabhu et al., 2018a) were required to maintain the Mn status of rainbow trout juveniles. These two contrasting reports further reiterates that the Mn inclusion levels in plant-based diet formulation for salmonids are largely determined by the combination of ingredients used. The present study showed that in post-smolt Atlantic salmon, Mn supplementation (as $MnSO_4$) of 14.1 to 22.1 mg kg^{-1} was required in low fish meal, plant-based basal diets containing 15 mg Mn kg^{-1}. As discussed earlier, Mn requirement (Maage et al., 2000) or dietary Mn level needed to meet the requirement of Atlantic salmon when fed practical fish meal diets (Lorentzen et al., 1996) have been established only during parr stage, which present close similarity with the reports in rainbow trout (Satoh et al., 1991) and other freshwater fish species (Antony Jesu Prabhu et al., 2016). The reports available on Mn requirement for marine fish species range between 12 and 25 mg kg^{-1} diet (Liu et al., 2013; Ye et al., 2009; Liu et al., 2018; Zhang et al., 2016; Nie et al., 2016). In the present study, although the requirement estimates (as total Mn) ranged between 30 and 37 mg kg^{-1} diet; corresponding Mn supplementation (as $MnSO_4$) levels were between 14.1 and 22.1 mg kg^{-1} diet. It is important to have in mind that the present study used a low fish meal practical diet, while the earlier cited studies used casein-based diets. The wide range of the recommendation also underlines the sensitivity of different criteria used to estimate dietary Mn requirement. The most common criterion used has been weight gain or other growth indices (Antony Jesu Prabhu et al., 2016). However, growth was not differentially affected by Mn levels in the present study. The fish were > 300 g at start, thereby reducing the sensitivity of growth as a criterion to assess dietary mineral requirement, as shown for phosphorus by Hardy et al. (1993). Literature based metadata analysis showed vertebrae Mn concentration to be the most robust criterion, with 70% and 45% higher requirement than for weight gain and whole body Mn concentration as criteria, respectively (Antony Jesu Prabhu et al., 2016). Although weight gain was non-responsive in the present study, the estimate obtained with vertebrate Mn concentration as criterion was 35% higher than obtained through whole body Mn concentration (22.1 vs. 16.3 mg Mn supplementation kg^{-1} diet).

The entero-hepato-biliary pathway plays a vital physiological role in trace metal homeostasis, including Mn (Hambidge, 2003), a better understanding of which can enable identification of pertinent biomarkers to assess trace metal status, bioavailability, interactions and potential toxicity (Hauser-Davis et al., 2012). Once absorbed at the intestine, Mn is transported by the blood to be either transformed in the liver and subsequently stored (e.g. bone); or excreted through bile which is defecated along with faeces (Nussey et al., 2000). A small fraction of biliary Mn excreted into the intestine is reabsorbed, establishing *de facto* enterohepatic circulation and the rest excreted in the faeces (Schroeder et al., 1966). Gastrointestinal absorption and biliary elimination of Mn, the two main regulatory sites of Mn homeostasis, are influenced by the dietary Mn intake (Aschner and Aschner, 2005; Britton and Cotzias, 1966). Reduced intestinal absorption, enhanced liver metabolism, and increased biliary excretion are adaptive mechanisms during high dietary intake of Mn (Aschner and Aschner, 2005). Understanding of the physiological aspects of Mn metabolism are rudimentary in fish. Whole body and vertebrae Mn concentrations are long term indicators of Mn status in fish (Satoh et al., 1987; Yamamoto et al., 1983) and responded with significant dietary effects at the end of 8 weeks. On the other hand, plasma and bile Mn concentrations were found to be responsive even at week 4 and the effects persisted until week 8. Among different criteria used to study Mn nutrition and determine Mn requirement in fish, Mn concentrations in plasma and bile were the least and seldom studied, respectively (Antony Jesu Prabhu et al., 2016). In our study, dynamic changes in entero-hepato-biliary Mn metabolism as early biomarkers for determining dietary Mn availability and requirement in Atlantic salmon were recorded. Plasma Mn concentration peaked in fish fed with 15 mg

Table 9
Relative bioavailability index of Mn-glycine over $MnSO_4$ to meet the Mn requirement of Atlantic salmon in seawater fed low fish meal, plant-based ingredients diets.

Criteria	A: Saturation level reached (mg kg^{-1} wet weight or μM)	B: Mn (as MnSO4) required to reach saturation (mg kg^{-1} diet)	C: Response level reached with 5 mg Mn kg^{-1} Mn-gly (mg kg^{-1} wet weight or μM)	D: Percentage saturation with 5 mg Mn kg^{-1} Mn-gly	E: Relative bioavailability index	F: Estimated inclusion of Mn-gly to meet requirement
Whole body Mn	1.7	16.3	1.5 ^{ns}	88.2	2.9	5.7
Plasma Mn	3.02	14.1	2.8 ^{ns}	92.7	2.6	5.4
Vertebrae Mn	13.4	22.1	13.7 ^{ns}	102.2	4.5	4.9

(A) Saturation level reached: mean value of parameter L (plateau) from the non-linear regression model presented in Fig. 1.
(B) Mn (as MnSO4) required to reach saturation: mean value of parameter R from the non-linear regression model presented in Fig. 1.
(C) Response level reached with 5 mg Mn kg^{-1} Mn-gly: mean value of data presented for 5 mg Mn kg^{-1} Mn-gly group in Tables 4 and 5; superscript denotes the lack of significant difference between C and A.
(D) Percentage saturation with 5 mg Mn kg^{-1} Mn-gly: calculated as (C/A) *100.
(E) Relative bioavailability index: calculated as (B/5) * (C/A).
(F) Estimated inclusion of Mn-gly to meet requirement: calculated as B/E.

Mn kg^{-1} supplementation (as $MnSO_4$) whereas, the peak in bile Mn was observed in fish fed with 35 mg Mn kg^{-1} supplemented diet as $MnSO_4$, indicating a dietary Mn intake driven homeostatic regulation along the hepatobiliary system (plasma-liver-bile). Atlantic salmon fed diets supplemented in excess of 15 mg Mn kg^{-1} as $MnSO_4$ responded with significant increase in liver, bile and faeces Mn concentrations. During excess Mn intake in higher vertebrates, excess Mn is removed from plasma by liver and eliminated through bile (Aschner and Aschner, 2005), maintaining a stable plasma to bile ratio of Mn (Klaassen, 1974). In the present study, highly significant correlations between well-established markers of Mn status in fish like whole body and tissue Mn concentration (Shearer, 1995; Antony Jesu Prabhu et al., 2016) and excretion of Mn in bile and faeces were also observed. Taken together, biomarkers related to entero-hepato-biliary pathway were better early indicators of Mn status; based on which, 15 mg kg^{-1} supplementation as $MnSO_4$ is recommended to low fish meal, plant-based ingredients diets in Atlantic salmon.

One of the major factor limiting the availability of Mn, and other trace minerals in salmonid feeds with high inclusion of plant-based ingredients is phytic acid (Storebakken et al., 1998). Phytic acid, the predominant storage form of phosphorus in grains, has high affinity to chelate other divalent metals ions such as Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} (Nolan et al., 1987), and reduce their availability to salmonids (Overturf et al., 2003). Unlike inorganic trace mineral sources, organic mineral forms have the potential to avoid negative interactions with anti-nutrients like phytic acid (Apines-Amar et al., 2004). Further, trace minerals chelated with amino acid ligands can also facilitate alternate intestinal uptake routes (Antony Jesu Prabhu et al., 2018b). Manganese supplied as 2-hydroxy-4-(methylthio)butyrate (HMB) or Mn-gly was found to be more efficient as dietary Mn source compared to $MnSO_4$ in cobia and turbot (Nie et al., 2016; Ma et al., 2015). However, here and in our previous study (Silva et al., 2019), AAC of Mn-gly were lower than $MnSO_4$ in post-smolt Atlantic salmon. Biliary secretion is the main pathway for Mn excretion; in rats fed diet containing 45 mg of Mn kg^{-1}, 8.2% of their Mn intake was absorbed whilst 37% of which was subsequently re-excreted in the faeces (Davis et al., 1993). Therefore, AAC as a response variable to assess absorption efficiency of dietary Mn can lead to an underestimation and explains in part the low (or negative) values reported in our previous study (Silva et al., 2019) and in literature (Sugiura et al., 1998).

The rational and benefit of using organic mineral sources in fish feeds is to minimise effects of anti-nutrients, improve bioavailability, satisfy requirement with lower supplementation levels than required with inorganic sources and ultimately reduce environmental load. Inclusion of 5 mg Mn kg^{-1} as Mn-gly was able to elicit response in whole body, plasma and vertebrae Mn concertation on par with 15 mg Mn kg^{-1} inclusion as $MnSO_4$. On the contrary, inclusion of 15 mg Mn kg^{-1} diet as Mn-gly significantly reduced plasma Mn and increased faecal Mn loss. Moreover, it also reduced the AAC of Zn and Cu; and Zn concentration in plasma, liver and vertebrae. Most of the essential trace elements known in fish are divalent metal ions (Zn^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+}), which may compete for binding sites in divalent metal transport system at the apical membrane (Bury et al., 2003). In rainbow trout, low dietary supply of Mn reduced hepatic mineral levels including Zn (Knox et al., 1981), which was also observed in this study to low Mn supplementation as $MnSO_4$ and high supplementation as Mn-gly. It is therefore important to understand the interactions between micro-minerals, their sources and intake levels, as the metabolic handling of a mineral source may not be independent of the dietary concentrations.

Relative bioavailability index calculated based on slope-ratio or response saturation have proven effective to assess the efficacy of alternate mineral sources (Paripatananont and Lovell, 1997). In the study of Silva et al. (2019), Mn-gly supplementation at 10 mg Mn kg^{-1} diet resulted in significantly lower AAC values for Mn-gly, like those observed for 15 mg Mn kg^{-1} diet as Mn-gly in this study. However, the Mn-gly source at 5 mg Mn kg^{-1} supplementation used in the present

study was found to be 2.6 to 4.5-fold more efficient compared to $MnSO_4$ in maintaining Mn status, which also resulted in better retention of absorbed Mn and overall growth performance. On the contrary, higher supplementation of Mn-gly (15 mg Mn kg^{-1}) resulted in significantly low plasma Mn and lowest growth (statistically non-significant). Therefore, while examining the efficacy of dietary Mn sources, it is important to consider the interaction between Mn source and level of inclusion. In conclusion, Atlantic salmon in seawater fed low fish meal, plant-based ingredients diets require Mn supplementation in range of 14 to 21 mg Mn kg^{-1} as $MnSO_4$. Our data suggests that, by using lower supplementation levels of 4.9 to 5.7 mg Mn kg^{-1} diet as Mn-gly, it will be possible to achieve similar response levels without compromising the growth, Mn status of tissues and whole body of Atlantic salmon and significantly reducing environmental Mn load.

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Appendix A. Supplementary data

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